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2nd Chair and Department of General Surgery, Medical University of Lublin
Chair and Department of Pathomorphology, Medical University of Lublin

ANDRZEJ DĄBROWSKI, JUSTYNA SZUMIŁO,
GRZEGORZ BRAJERSKI, GRZEGORZ WALLNER

Proliferating nuclear antigen (PCNA) as a prognostic factor of squamous cell carcinoma of the oesophagus

The loss of control over proliferation is one of the main causes of transformation of a normal cell into a malignant cell. Considerable progress in evaluation of proliferating activity of cells was made possible by immunohistochemical methods (7).

Proliferating cell nuclear antigen PCNA was detected and described by M i y a c h i and co-workers in 1978 in blood serum of patients affected with systemic lupus erythematosus (8). This polypeptide was then localised in nucleuses of proliferating cells. PCNA expression increases considerably in the G1 phase of the cell cycle and acquires its culminating point in the S phase (15).The characteristic feature of PCNA protein is relatively long period of semiduration which equals about 20 hours. (5).

Proliferating cell nuclear antigen PCNA identified as p36 protein takes part both in replication as well as repair of DNA (4). It plays the role of a cofactor of DNA delta polymerase (10). Direct influence of PCNA and p21 protein was also found. It is assumed that mechanism of p21 protein activity depends on the time in which damage of DNA takes place. In case of DNA damage before the beginning of the S phase, p21 protein would act as inhibitor of cdk kinases. If DNA damage took place during the S phase, then p21 protein, combining with PCNA, would block replication of DNA (4).

The aim of the performed tests was establishing whether there exists relation between the value of PCNA proliferating index evaluated in oesophageal squamous cell carcinomas and the depth of malignant infiltration, invasion of lymph nodes, presence of metastases in extraregional lymph nodes and the length of patients' survival.

MATERIAL AND METHODS

The proliferative activity of PCNA underwent analysis in bioptic material from 60 patients with oesophageal squamous cell carcinoma, localised in the thoracic section.These patients were operated in the years 1992-1995 in the Second Chair and Department of Medical University, Lublin, Poland. Among 60 patients there were 54 (90%) men and 6 (10%) women (male to female ratio 9:1). Age of patients ranged from 43 to 70 years; on average 57.9± 8.0 years. On the basis of such tests as:

radiological, endoscopic (in chosen cases endoscopic ultrasonography – EUS), ultrasonographic – USG examination and computer tomography – KT, the degree of clinical progression of the neoplasm was established. The basis of its determination was UICC classification of 1987 (7). With the exception of 10 patients, the remaining ones overused alcohol and were cigarette smokers for many years. Forty patients from this group were subjected to chemotherapy or chemo- and radiotherapy before the operation.

Histopathological and immunohistochemical examinations were performed at the Chair and Department of Pathomorphology of Medical University, Lublin. Both endoscopic and surgical specimens were fixed in 10% buffered formalin for a period not longer than 24 hours. During endoscopy 3 to 6 specimens of oesophageal carcinoma were collected. The following samples were obtained routinely from surgical material: taenia from tumour (3 to 8 specimens), taenia of normal tissue from the oesophagus (2 specimens) and from the stomach (1 specimen), cutting margins and lymph nodes of appropriate groups. Collected specimens were embedded in paraffin. Sections having a thickness of 4 μm were cut, and later were stained with hematoxylin and eosin (H+E) and mucicarmine. In the histopathological examination the following features were determined: the infiltration depth of carcinoma in the oesophagus wall (pT factor), number of lymph nodes metastases and their distribution (pN factor), presence of distant metastases to extra-regional lymph nodes (M_{lym+} factor), thus degree of the carcinoma pathological progression (pTNM). Carcinoma macroscopic appearance, dimensions of a neoplastic focus and presence of neoplastic cells in the oesophageal vessels were also evaluated. Immunohistochemical reactions were performed on paraffin cuts from specimens obtained during the endoscopic examination.

AB Complex/HRP (the avidin-biotin complex marked with peroxidase) method was used for immunohistochemical examinations. In order to unmask antigen after removing paraffin and hydration, sections immersed in distilled water were placed in a microwave cooker (Samsung Electronics 750W) 2 times for 5 minutes. Endogenous peroxidase was blocked by incubating sections with 3% hydrogen peroxide for 20 minutes in room temperature. The sections were covered with Dako rabbit serum in dilution of 1:20 for 30 minutes in room temperature. Then sections were incubated with monoclononal mouse antigen against proliferating cell nuclear antigen PCNA (PC 10m Dako clone) in dilution of 1:50 overnight in the temperature of 4^0 C. In the next stage the secondary rabbit antigen was used (Dako) in dilution 1:300 and the avidin-biotin complex marked with peroxidase (Dako) for 30 minutes in room temperature. Stain reactions were caused by incubating sections with a solution of uterochloride 3,3'-diaminobenzidine (DAB –Dako).

Each time while performing immunohistochemical reactions, positive and negative control was used. In negative controls primary antigens were replaced with company control mice serums (mouse IgG1 No. X 0943). Positive controls constituted sections with colorectal adenocarcinoma, showing intensive positive reaction with the examined antigen.

Semi-quantitative evaluation of PCNA expression was performed. In the fields of the highest PCNA expression under magnification of 250 times, percentage of cells showing positive reaction in 1,000 neoplastic cells (a Jenamed 2 microscope of Carl Zeiss Jena Co.) was counted. Only nuclear reaction was taken into consideration irrespective of the degree of staining intensity. PCNA index was considered low when <50% of cells showing the positive reaction was present, and high when there were >50% of these cells.

METHODS OF STATISTICAL ANALYSIS

In order to demonstrate relations and differences referring to this study a series of statistical methods was used. Differences between averages were tested with the help of t-Student test. From analysis of survival time patients who died in the postoperative period because of complications (postoperative mortality), were excluded. Survival curves were constructed with the help of the Kaplan-Meier method. The influence of examined parameters on survival was evaluated using the Cox's model of proportional risk. The influence of parameters on the occurrence or nonoccurrence of a given phenomenon was assessed using the logistic regression model, because many examined parameters did not have normal distribution. Many non-parametric tests were used for this analysis such as: Kruskal-Wallis, Sperman and Kendal's non-parametric correlation. The $p=0.05$ value was assumed as statistically significant.

RESULTS

It was shown that in 7 cases (11.6%) the tumour was localised in a third upper part of the thoracic oesophagus, in 40 patients (66.6%) in the middle part and in 13 cases (21.6%) in the lower part. Radiological examination enabled to determine a type of the neoplastic infiltration. The most often observed type of the funnel-shaped tumour was discovered in 25 (41.7%) patients, the spiral type in 21 (35%) patients, the toothed type in 10 (16.7%) patients and the tumour-like type in 4 (6.7%) examined patients. In the endoscopic picture neoplastic infiltration was defined as the diffuse-infiltrative in 23 (38.3%) of patients, in 19 (31.7%) the ulcerative and infiltrative one, in 10 (16.7%) prominent, whereas in 8 (13.3%) the ulcerative and non-infiltrative one. On the basis of the TNM classification it was considered that 19 (31.6%) patients were in the second stage, while the remaining 41 (68.3%) in the third stage of carcinoma advancement. Squamous cell highly differentiated carcinoma was diagnosed at 8 (13.3%) patients, medium differentiated carcinoma in 29 (48.3%) and low differentiated carcinoma at following 18 (30%) patients. In 5 cases no neoplastic cells were found in the surgical specimen – pT0 factor. These were patients previously subjected to chemotherapy, in whom complete response or mixed effect to the treatment was obtained, i.e. complete response in the primary focus in the oesophagus and simultaneous presence of metastases in lymph nodes. In 2 (3.3%) cases infiltration was limited to the submucosa – pT1 factor, in 10 (16.6%) it covered the muscle layer – pT2 factor, whereas in 25 cases (41.6%) it infiltrated the whole wall – pT3 factor, while at the remaining 18 patients (30%) it covered tissues adhering the oesophagus – pT4 factor. The presence of metastases in the regional lymph nodes was discovered in 34 (56.6%), in the remaining 26 (43.3%) sick neoplastic cells were not discovered. Moreover, in 11 cases (18.3%) metastases in extra-regional lymph were present. Progression degree 0 (in patients with complete response to initial treatment) it was discovered in 3 cases (5%), I – at 2 (3.3%), IIa – in 15 (25%), IIb – 3 (5%), III – in 26 (43.3%) and IV – in 11 (18.3%) cases. Neoplastic cells in vessel area were discovered in 33 (55%) cases.

Reaction to PCNA was the granular-spread out nuclear one. It showed heterogeneity in respect to staining intensity of cell nucleuses – from dark brown (strongly positive reaction) to light brown (weakly positive reaction). All cells of squamous cell carcinoma showing nuclear reaction irrespective of the staining intensity degree, were considered positive. In some cases negative reaction was found in nucleoluses range. Neoplastic cells showing PCNA expression were distributed irregularly. Fields having high antigen expression (chosen for examination) were adjacent to fields having low expression. The highest PCNA expression was usually discovered in circumferential part of neoplastic texture, on

the border with normal tissue and in tiny carcinoma focuses in places of the deepest infiltration. Positive reaction was also observed in cells of the parabasal layer of the stratified squamous noncornifying epithelium of the oesophagus and focally in reproduction centres of lymphatic focilles, in cells of the secretory part of proper oesophageal nodules, in fibroblasts and endothelial cells of vessels.

Dependence between PCNA index and features: pT, pN, pM_{lym} presents Table 1. PCNA index appeared to be prognostically useful for evaluation of metastases to lymph nodes ($p<0.04$) , which was also reflected in pTNM classification ($p>0.01$). Tendency to higher values of PCNA index was observed in patients with more progressed neoplasm. This marker did not correlate with pM_{lym+} factor. Simultaneously the existence of the significant correlation between vessels infiltration in the oesophagus wall and value of PCNA index was found ($p<0.001$).

Table 1. Correlation between chosen morphological data and PCNA index evaluation in biopsy specimens (n = 60)

Presence of metastases in extra-regional lymph nodes (pM_{lym})		ns	
pM_{lym-}	29 (82.9%)	20 (80%)	
pM_{lym+}	6 (17.1%)	5 (20%)	
Histopathological examination of surgical specimens		ns	
1. Neoplastic texture was not discovered	3 (9.7%)	2 (6.9%)	
2. Highly differentiated ca planop.	3 (9.7%)	5(17.2%)	
3. Medium differentiated ca planop.	13 (41.9%)	16 (55.2%)	
4. Lowly differentiated ca planop.	12 (38.7%)	6 (20.7%)	
Infiltration depth – pT			ns
pT0	3 (9.7%)	2 (6.9%)	
pT1	2 (6.5%)	0	
pT2	7 (22.6%)	3 (10.3%)	
pT3	12 (38.7%)	13 (44.8%)	
pT4	7 (22.6%)	11 (37.9%)	
Evaluation of lymph nodes pN			p<0.04
0 – N0	18 (58.1%)	8 (27.6%)	
1 – N1	13 (41.9%)	21 (72.4%)	
Progression degree – pTNM			p<0.01
0*	2 (6.5%)	1 (3.4%)	
I	2 (6.5%)	0	
IIa	12 (38.7%)	3 (10.3%)	
IIb	1 (3.2%)	2 (6.9%)	
III	11 (35.5%)	15 (51.7%)	
IV	3 (9.7%)	8 (27.6%)	
Vessel infiltration			p<0.001
1 – yes	12 (38.7%)	21 (72.4%)	
2 – no	19 (61.3%)	8 (27.6%)	

* Patients in whom total response or mixed effect to initial treatment was obtained.

Prognostic values of PCNA index as a survival time marker, examined in preoperative biopsy specimens, are presented in Figure 1, for drawing of which the Cox's model of proportional risk was used. Survival of patients with neoplasms with a high (>50%) and low (<50%) PCNA index was not significantly different.

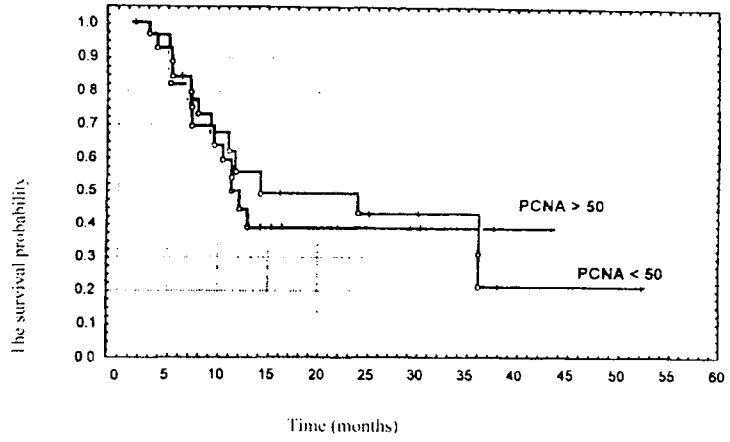

Fig. 1. The survival of patients with oesophageal squamous cell carcinomas and PCNA index preoperatively

DISCUSSION

Information about cell kinetics may be a useful addition to the tumour classification based first of all on histological evaluation and can contribute to understanding of a series of processes that take place in it (2, 5, 7, 10, 13). Manifestation of morphological functional condition of cells is their proliferating activity (12). Loss of control over proliferation is one of the main causes of transformation of a normal cell to a neoplastic one (15). Manifestation of irregularity of this process are changes of proliferation tempo of particular cell populations, changes in expression of proliferating antigens, various degree of morphological and functional differentiation of neoplasm (20). PCNA proliferating activity induced interest of researchers dealing with pathology of oesophageal squamous cell carcinoma (6, 7,12, 13, 15). It is caused by – admittedly few – but encouraging results acquired by some researchers discovering that determination of PCNA index can prove useful as a prognostic factor in oesophageal carcinoma (12, 13).

W a n g and co-workers subjected to examinations biopsy specimens obtained from 237 patients with oesophageal and gastric cardia cancers (15). These researchers on the basis of the obtained results came into conclusion that the proliferating PCNA index correlated well with the histopathological evaluation of oesophageal and stomach cardia carcinoma. In a normal oesophagus PCNA immunoreactivity was observed mainly in cells of the basal and parabasal layer, especially in verruca regions. In cases in which a sequence of changes was observed from hyperplasia through dysplasia to carcinoma, PCNA immunoreactivity increased 6 times and was observed also in upper epithelium layers. The mentioned authors also observed high correlation between excessive accumulation of p53

protein and PCNA index. Parallelly to showing up carcinoma focuses, there increased the phenomenon of excessive p53 protein accumulation and the number of cell nucleuses showing positive reaction to PCNA.

When interpreting the obtained results, a series of factors should be taken into consideration, which could have influence on their final conclusions. Many authors' opinions differ as far as the influence of fixing on reaction intensity to PCNA is concerned. H a e r s l e v and co-workers think that using a microwave cooker strengthens the reaction intensity and makes possible the usage of larger dilutions of antigen (4). According to H a l l and co-workers PC10 antigen recognises PCNA epithop resistant to fixing in formalin, therefore tissues fixed routinely in buffered formalin can be subjected to examination (5). However, C a s a s c o and co-workers consider that even short fixing decisively decreases expression and it cannot be restored using enzymatic digestion or acidic hydrolysis (1). N a k a n o and co-workers' experience shows that the optimal fixing time equals 24 hours (11). In the technique of specimen preparation the role of the unmasking method is also exalted (a way of cross bindings removal). Attention is also paid to the role of temperature as a factor taking place in denaturation of a DNA double filum to a single one in order to enable antigen approach to proteins bonded with DNA. Errors resulting from a subjective evaluation of the analysed specimens by the researchers, should be also taken into consideration (12, 13).

When evaluating the obtained reactions we discovered: instability of intensity from small to large and variety of staining from granular to spread out one. The observed heterogeneity of PCNA expression could account for differences in proliferation tempo of the particular portions of neoplastic texture. On the other hand, it could also result from repair processes of damaged DNA taking place simultaneously, which could appear on one of stages of the neoplasm transformation process. Therefore PCNA expression can be the result of two, so far known PCNA functions. Another feature influencing expression can be mRNA PCNA stabilisation, which undergoes translation, nevertheless actual RNA transcription processes do not take place in a given cell (5). There can be several causes of such a state of things. One of them can depend on damage of 4 introne responsible for regulation of PCNA concentration in non-proliferating cells (5). The second reason can be mRNA PCNA stimulation and of protein itself by growth features of PDGF and EGFR type (5, 6). The following features may be connected with authocrinnic influence of tumour, as well as long semi-duration period of PCNA, at the same time by its presence in cells which ended the cell cycle (5). In the light of presented interpretations it cannot be excluded that one or several mentioned mechanisms influenced results obtained by us. It is not also precluded that difference in acquired values of PCNA index was connected with a kind of specimens sampled for examination.

Determination of the prognostic value of PCNA proliferating index is connected with establishing a border value of this parameter. Therefore H i c k e y and co-workers assumed the index value over 40% of positively stained nucleuses in the analyses specimens from 14 patients for manifestation of PCNA expression (6). However, K u w a n o and co-workers (7) assumed the border value of PCNA index in 35% of positively stained nucleuses. Values not exceeding 35% were considered by authors as the low PCNA index, whereas index exceeding this value was considered high. The mentioned researchers evaluating age, sex, neoplasm location, infiltration depth, malignant progression of lymphatic and blood vessels, presence of metastases and neoplasm histological type could not prove correlation between these parameters and assumed values of the so-called low and high degrees of PCNA proliferation indexes.

In examinations carried out by us because of obtaining high average values of PCNA index the evaluated cases were classified as a group of high value (PCNA index level >50%) and a group of low value (PCNA index level <50%). At the same time a tendency was observed towards the appearance of PCNA index higher values as neoplasm progression increased.

In Hickey and co-workers' opinion high values of PCNA index can denote the appearance of metastases in the lymphatic system (6). Mori and co-workers certified that opinion and proved good correlation between PCNA index value and infiltration of the lymphatic system and blood vessels by neoplasm (9). But also in this case analysis related to the majority (8/12) of patients, in whom neoplastic infiltration did not exceed the submucosa. Other researchers had different opinions. They did not find any correlation between PCNA proliferation index values and neoplasm infiltration of the lymphatic system (nodes and vessels) (10, 13). Levels of PCNA proliferation indexes obtained by the authors of the hereby study, were essentially higher in preoperative evaluation ($p<0.04$) in case of neoplasm infiltration of lymphatic nodes and blood vessels in the oesophagus wall ($p<0.01$). In the analysis of the survival time of patients with tumours having a high PCNA index and a low PCNA index, no essential differences in survival length of both these groups were found. Although there exist different opinions as far as usefulness of an examined parameter is concerned in relation to particular clinical and pathological parameters, accordance seems to prevail that high values of PCNA index can be interpreted as existence of high infiltration readiness of the oesophageal carcinoma and prognosis in such cases is unfavourable (6, 7, 9).

In some reports concerning the use of PCNA index as a prognostic marker in oesophageal squamous cell carcinoma, researchers' caution in formulating final conclusions attracts attention. Caution in interpretation of results is undoubtedly connected with many difficulties, which we tried to signalize in the hereby elaboration.

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SUMMARY

Proliferating nuclear antigen (PCNA) plays an important role both in the process of replication and repair of DNA. The aim of the carried out investigations was to find out if there was any correlation between the depth of malignant infiltration, invasion of lymph nodes, presence of metastases in extraregional lymph nodes, the length of patients' survival, and the value of PCNA proliferating index analysed in biopsy specimens before the operation, taken from 60 patients with oesophageal squamous cell carcinoma.

In the groups with advanced squamous cell carcinoma of the oesophagus the following statistic correlation was found between PCNA proliferating index and: invasion of lymph nodes – $p< 0.04$; degree of carcinoma progression – $p< 0.01$; and blood vessels infiltration – $p< 0.001$. The survival of patients with carcinomas of high (> 50%) and low (< 50%) PCNA index did not differ significantly.

It results from the above investigations that high values of PCNA index may indicate the presence of metastases in lymphatic nodes and the degree of progression of oesophageal carcinoma.

Proliferacyjny antygen jądrowy (PCNA) jako czynnik prognostyczny u chorych z płaskonabłonkowym rakiem przełyku

Proliferacyjny antygen jądrowy (PCNA) pełni ważną rolę zarówno w procesie replikacji jak i naprawy DNA. Celem przeprowadzonych badań było ustalenie, czy istnieje związek pomiędzy głębokością nacieku nowotworowego, zajęciem węzłów chłonnych, obecnością przerzutów w pozaregionalnych węzłach chłonnych, długością przeżycia chorych, a wartością indeksu proliferacyjnego PCNA badanego w materiale biopsyjnym przed operacją u 60 chorych z płaskonabłonkowym rakiem przełyku.

W grupach z zaawansowanym rakiem przełyku wykazano następującą zależność statystyczną pomiędzy indeksem PCNA a: obecnością przerzutów do węzłów chłonnych – $p<0.04$, stopniem

zaawansowania raka – $p<0.01$ i naciekaniem naczyń – $p<0.001$. Przeżycie chorych z nowotworami o wysokim (>50 %) oraz o niskim indeksie PCNA (<50 %) nie różniło się istotnie.

Z przeprowadzonych badań wynika, że stwierdzenie wysokich wartości indeksu PCNA może wskazywać na obecność przerzutów do układu chłonnego i stopień zaawansowania raka przełyku.